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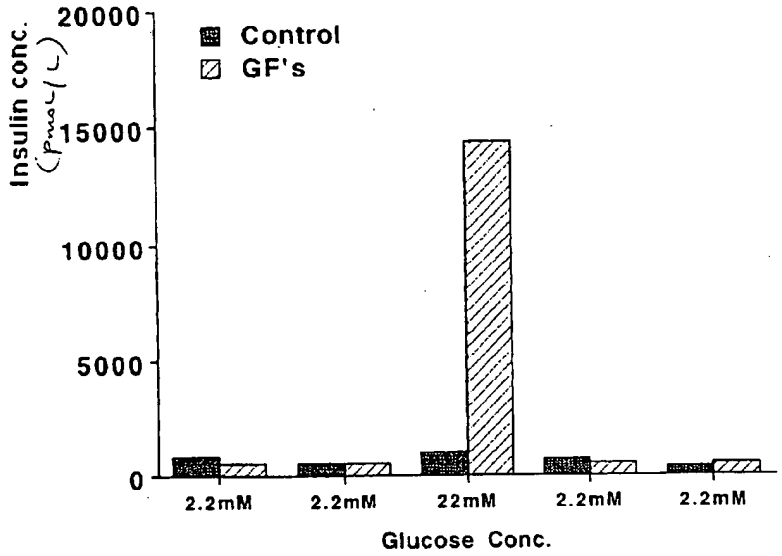
(54) **MILIEU FAVORISANT LA SURVIE DES CELLULES SECRETRICES D'INSULINE**

(54) **A MEDIUM TO PROMOTE ISLET CELL SURVIVAL**



Stimulation of insulin secretion by a glucose challenge in-vitro. Note the significant increase in insulin response for those islets incubated in NGF + IGF-1

(57) L'invention concerne un milieu de culture favorisant la survie des cellules sécrétrices d'insuline de mammifères, qui comprend au moins une quantité efficace d'un ou de plusieurs facteurs de croissance ayant un effet anti-apoptose sur les cellules sécrétrices d'insuline dans un milieu de culture physiologique.

(57) The present invention relates to a culture medium for promoting the survival of mammalian islet cells, which comprises at least an effective amount of one or more growth factor having anti-apoptosis effect on islet cells in a physiologically acceptable culture medium.

ABSTRACT OF THE INVENTION

The present invention relates to a culture medium for promoting the survival of mammalian islet cells, which comprises at least an effective amount of one or more growth factor having anti-apoptosis effect on islet cells in a physiologically acceptable culture medium.

A MEDIUM TO PROMOTE ISLET CELL SURVIVAL

BACKGROUND OF THE INVENTION

(a) Field of the Invention

The invention relates to a culture medium which promote islet cell survival, which may be transplanted to reverse hyperglycemia.

(b) Description of Prior Art

Adequate numbers of isogeneic islets transplanted into a reliable implantation site can only reverse the metabolic abnormalities in diabetic recipients in the short term. In those that were normoglycemic post-transplant, hyperglycemia recurred within 3-12 months. The return of the diabetic state that occurs with time has been attributed either to the ectopic location of the islets, to a disruption of the enteroinsular axis, or to the transplantation of an inadequate islet cell mass.

Studies of the long term natural history of the islet transplant, that examine parameters other than graft function, are few in number. Only one report was found in which an attempt was specifically made to study graft morphology (Alejandro R. et al., *J. Clin. Invest.*, 1986, **78**:1339). In that study, purified islets were transplanted into the canine liver via the portal vein. During prolonged follow-up, delayed failures of graft function occurred. Unfortunately, the graft was only examined at the end of the study, and not over time as function declined. Delayed graft failures have also been confirmed by other investigators for dogs and primates. Most failures are presumed to be the result of rejection despite appropriate immunosuppression.

Because of these failures, there is currently much enthusiasm for the immunoisolation of islets,

which could eliminate the need for immunosuppression. The reasons are compelling. Immunosuppression is harmful to the recipient, and may impair islet function and possibly cell survival. Unfortunately, micro-encapsulated islets injected into the peritoneal cavity of the dog fail within 6 months (Soon-Shiong P. et al., *Transplantation*, 1992, **54**:769), and islets placed into a vascularized biohybrid pancreas also fail, but at about one year (Lanza RP et al., *Diabetes*, 1992, **41**:1503). Histological evaluation indicates a substantial loss of islet mass in these devices. No reasons have been advanced for these changes.

Only whole pancreas transplantation is capable of permanently reversing the diabetic state and preventing the secondary complications of diabetes. This suggests that there is a fundamental biological difference between grafts of whole pancreas and those of purified islets.

It is perhaps significant that the major emphasis in islet transplantation has been the enhancement of the purity of the islet preparation to promote engraftment and reduce immunogenicity. After more than 20 years of concerted research and more than 200 attempts at human islet transplantation, the achievement of insulin independence remains elusive. Contamination of the islet preparation by non-endocrine cells does not impair function or engraftment, nor enhance immunogenicity. In a canine model, pancreatic tissue fragments autotransplanted into the spleen appeared to survive better than islets refluxed into the liver (Kretemom NM and Warnock GL, *Transplantation*, 1990, **49**, 679). The survival advantage was attributed to the specific site, but pancreatic fragments may actually have an inherent survival advantage compared to purified islets. In people, insulin-independence

has been obtained after intraportal injection of unpurified autologous islets. Fewer impure islets were more successful than many more purified ones. A similar experience was repeated with allotransplants, with unpurified islets from a single human pancreas successfully reversing hyperglycemia (Gores PF et al., *Lancet*, 1993, **341**:19). From these and other reports, it is apparent that unpurified islets from one pancreas may survive as well as pure islets prepared from multiple donors.

To date, the only known roles of apoptosis, or programmed cell death, in the regulation of islet cell mass are in the involution of β cell mass after pregnancy and perhaps in remodeling during development as recently suggested by Finegood (Finegood OT et al., *Diabetes*, 1995, **44**:249). These situations, however, are physiologic in nature and it remains to be determined under what unusual circumstances islets may also be induced to undergo apoptotic cell death. The recent report of a selective decrease in β cell mass following transplantation of human islets into diabetic nude mice is therefore of considerable interest. The induction of apoptosis in relation to islet isolation and transplantation has not been reported previously. We have original observations on human islets after isolation. Light microscopic examination of these islets just prior to culture demonstrates that at least 15% of the cells have morphological evidence of apoptosis (pyknotic nuclei). Tissue transglutaminase (TG) expression is a fundamental event in the induction of apoptosis. TG is a calcium-dependent enzyme whose activity is well established in many mammalian tissues, including pancreas. It is involved in the cross-linking of intracellular proteins that precedes the irreversible ultrastructural changes characterizing

cells undergoing apoptosis. The appearance of apoptotic bodies parallels an increased expression of TG. Our pilot studies have shown TG activity to be elevated immediately following islet isolation, with continued increase up to at least 1 week. These findings correlated with data from a cell death ELISA that detects histone-associated DNA fragments, another characteristic of the apoptotic process (Paraskevas S. et al., *Transplant Proc.*, 1997, **29**:750). These fragments result from the activity of an endonuclease that cleaves DNA at internucleosomal sites. These data form the basis for investigating the role of apoptosis in islet survival after isolation.

It would be highly desirable to be provided with a means to prevent apoptosis of islet cells, thereby allowing transplantation with the successful reversal of hyperglycemia.

SUMMARY OF THE INVENTION

One aim of the present invention is to provide a culture medium which promote islet cell survival, which may be transplanted to reverse hyperglycemia.

In accordance with the present invention there is provided a culture medium for promoting the survival of mammalian islet cells, which comprises at least an effective amount of one or more growth factor having anti-apoptosis effect of islet cells in a physiologically acceptable culture medium.

The preferred growth factors include, without limitation, NGF, IGF-I and insulin. Other growth factors include TGFβ, IGF-II and HGF.

The preferred NGF concentration is between about 10 to about 100ng/ml of medium.

The preferred IGF-I concentration is between about 10 to about 100ng/ml of medium.

Preferably, the culture medium of the present invention includes a immunosuppressant.

The preferred immunosuppressants are selected from the group consisting of FK506 and a peptide having NGF potentiating effect, and therefore possibly a trophic effect on islet cells.

The preferred immunosuppressant concentration is about 1 micromolar.

More preferably, the culture medium of the present invention further include insulin. The preferred insulin concentration is from about 10 to about 100ng/ml of medium.

The term "physiologically acceptable culture medium" is intended to mean any commercially available culture medium including, without limitation, CMRL 1066, RAM 1640 and DMEM/F12.

The medium of the present invention may also be used to isolate islet cells or to irrigate the site of transplant to promote *in situ* islet cell survival.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates an Agarose gel demonstrating DNA laddering in islets incubated in a control medium (control lane), compared to islets incubated with insulin or NGF;

Fig. 2 illustrates TUNEL staining of porcine islets, (A) islets incubated in the standard medium and (B) islets incubated with insulin; and

Fig. 3 illustrates a graph of the stimulation of insulin secretion by a glucose challenge *in vitro*.

DETAILED DESCRIPTION OF THE INVENTION

Growth factors act in an autocrine and/or paracrine manner to mediate a broad range of cellular responses, including ECM formation, cell proliferation

and differentiation. The role of these factors in islet cell survival in the pancreas is relatively unexplored.

Hepatocyte growth factor (HGF) has effects on a wide variety of epithelial cell types, including pancreas, where it has been detected in acinar and islet cells. It has no known role in the adult pancreas. In comparison, Insulin-like growth factors (IGFs) effect cell growth and differentiation in both the fetal and adult pancreas. During induced islet cell neogenesis in the adult hamster, we have found that β cell differentiation from the ductal epithelium was associated with a transient increase in IGF-II expression, recapitulating the fetal environment as characterized by Hill (Rafacloff R et al., *Diabetes*, 1993, **42**(suppl.1):137A). This suggests a direct role for IGF-II in the differentiation process in the adult. Evidence for IGFs as survival factors has also been reported. Similarly, recent findings support a role for Nerve growth factor (NGF) in normal development, morphogenesis and functional maturation of islets. Greater uncertainty surrounds reg, a gene that is over-expressed within pancreatic cells after pancreatitis or resection, and particularly during islet cell regeneration. We and others have found that the reg gene is induced in our model of islet neogenesis. Although it has been hypothesized that reg is an important paracrine factor in the maintenance and growth of β-cells, the true significance of this gene remains to be determined. Transforming Growth Factor (TGF)-α is a mitogen expressed in pancreatic duct and acinar cells, whose role in maintaining cell survival is undefined. TGF-β is also a potent regulator of cell proliferation, but its major activity is to stimulate

the synthesis and deposition of various ECM proteins and to increase the expression of integrins. TGF-β expression *in vivo* may be important in the re-establishment of basement membrane following its loss during islet isolation, and hence in islet cell survival.

Growth factors may also be inhibitory in almost all situations in which apoptosis, or programmed cell death, is positively stimulated. This suggests that regulation of growth factor levels is not only important in the control of cell proliferation but also in maintaining viability of cells susceptible to apoptosis. These data suggest, that for the success of islet transplantation, the activity of survival factors may be fundamental to the long term maintenance of graft function.

The preferred Islet Survival Medium of the present invention

The formulation of the preferred medium of the present invention takes advantage of (1) newly recognized effects of known growth factors on inhibiting the induction of apoptosis in islet cells, (2) the combination of these factors to achieve a synergistic beneficial effect on islet cell survival, and (3) the potentiation of this synergistic effect by the addition of the drug FK506 (ProGraf®, Fujisawa).

The growth factors that have been combined are NGF (nerve growth factor), IGF-I (insulin-like growth factor-1) and insulin. None has been previously known to play a role in the prevention of apoptosis. Certainly, the role of NGF on islet tissue has only recently been recognized with respect to islet cell differentiation during normal fetal development. Its known trophic effects on cells have been solely in relation to neuronal cells. Similarly, IGF-I is active

in islet cell differentiation during fetal development, but a role in apoptosis has never been reported.

FK506 is a macrolide immunosuppressive agent which acts by inhibiting T-cell activation. FK506 is a ligand for the immunophilin FKBP12. The FK506-FKBP12 complex binds several targets, one of which is the calcium calmodulin dependent phosphatase, calcineurin. FK506 has been shown to potentiate the neurotropic effects of NGF. The exact mechanism by which FK506 facilitates NGF activity is still unclear.

We have demonstrated that a CMRL 1066 medium (a conventional islet culture medium) supplemented with the above substances can significantly reduce the amount of apoptotic cell death sustained by insulin-producing β-cells following islet isolation and purification.

The evidence is based on four assays.

First, Western blots demonstrate that the balance between activation of stress-activated protein kinases (SAPK) including JNK1/JNK2 and p38 relative to erk1/erk2, is altered. The former two pathways are known to be associated with the induction of the apoptotic program, while the latter may be protective.

Second, there is a significant reduction on agarose gels of the DNA laddering that is characteristic for the DNA fragmentation that is the outcome of apoptosis (Fig. 1).

Third, TUNEL staining (another technique to visualize apoptotic events) of actual isolated islets, demonstrates a significant reduction in the number of apoptotic cells (Fig. 2).

Finally, *in vitro* functional studies demonstrate that islets cultured in the presence of IGF-I, NGF and insulin have a much improved insulin

secretory response to a glucose challenge (the hallmark of β-cell function) (Fig. 3).

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. A culture medium for promoting the survival of mammalian islet cells, which comprises at least an effective amount of one or more growth factor having anti-apoptosis effect on islet cells in a physiologically acceptable culture medium.

2. The culture medium of claim 1, wherein said growth factors comprises NGF and IGF-I.

3. The culture medium of claim 2, wherein NGF is in a concentration of about 10 to about 100ng/ml of medium and IGF-I is in a concentration of about 10 to about 100ng/ml of medium.

4. The culture medium of claim 1, which further comprises an immunosuppressant.

5. The culture medium of claim 4, wherein said immunosuppressant is selected from the group consisting of FK506 and a peptide having NGF potentiating effect.

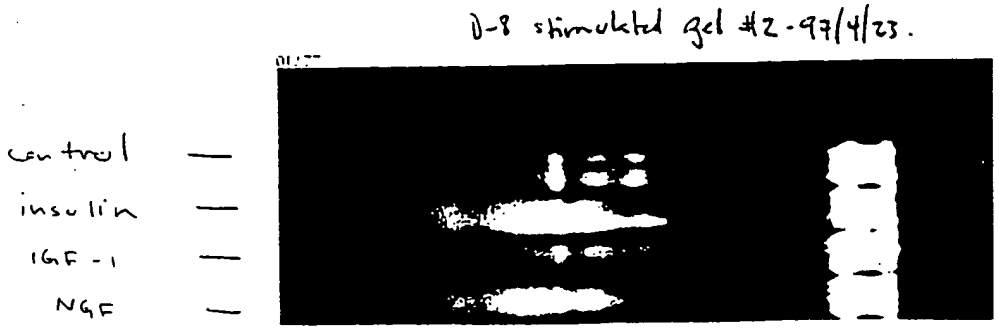
6. The culture medium of claim 5, wherein said immunosuppressant is in a concentration of about 1 micromolar.

7. The culture medium of claim 4, which further comprises insulin.

8. The culture medium of claim 7, which comprises insulin at a concentration of about 10 to about 100ng/ml of medium.

9. The culture medium of claim 7, wherein said growth factor is selected from the group consisting of TGFβ, IGF-II and HGF.

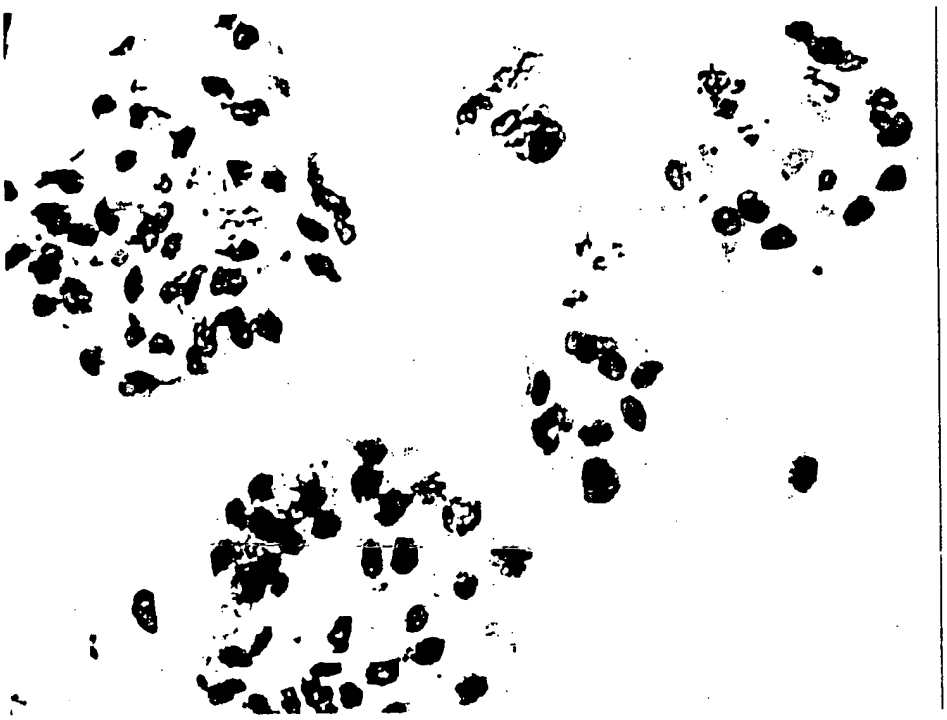
10. The culture medium of claim 9, wherein said physiologically acceptable culture medium is selected from the group consisting of CMRL 1066, RPMI 1640 and DMEM/F12.



Agarose gel demonstrating DNA laddering, a sign of apoptosis, in islets incubated in a control medium (control lane), compared to islets incubated with insulin or NGF

Fig. 1

A

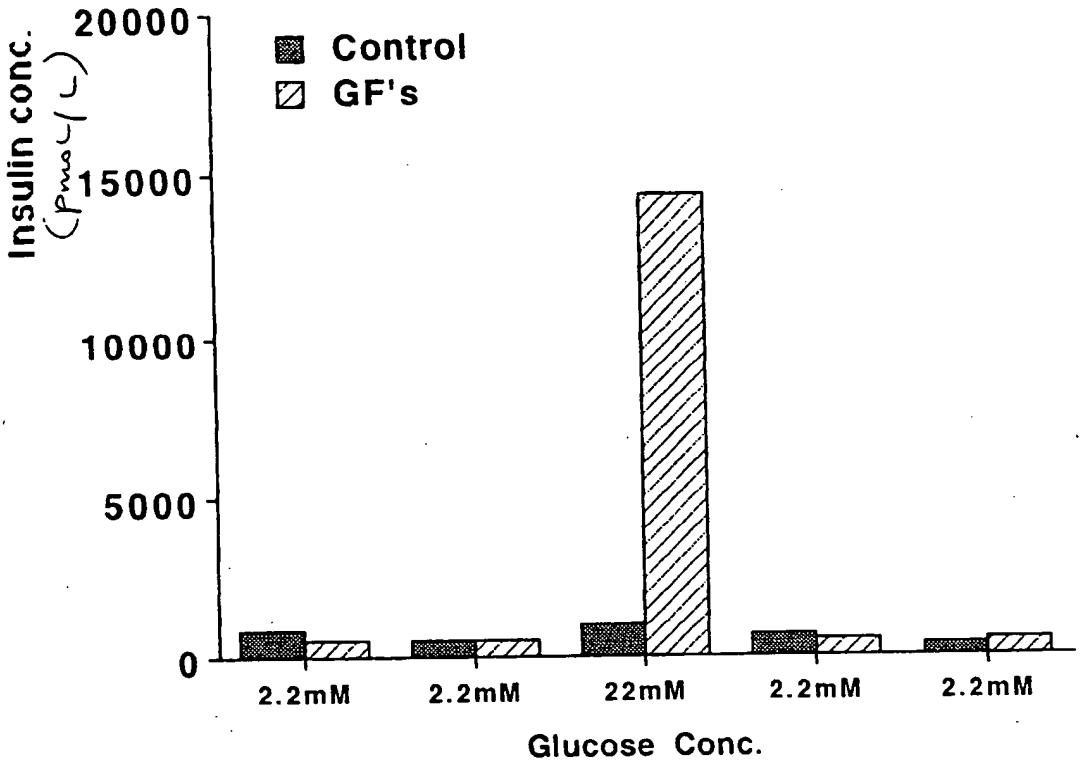


TUNEL staining of porcine islets. The TUNEL assay indicates which cells are apoptotic.

B

Islets in A represent islets incubated in the standard medium. Islets in B are islets that have been incubated with insulin.

Fig. 2



Stimulation of insulin secretion by a glucose challenge in-vitro. Note the significant increase in insulin response for those islets incubated in NGF + IGF-1

Fig. 3